BIOLOGY OF DIFFUSIBLE POLLEN WALL COMPOUNDS

By

Edward George Kirby, III

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1977

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ACKNOWLEDGEMENTS

I must express my sincere appreciation to Dr. Indra Vasil (committee chairman) for his guidance and meaningful discussions during the course of my studies. Also, I wish to express thanks to Dr. Willard W. Payne, committee cochairman; Dr. Henry C. Aldrich; Dr. Ray E. Goddard; and Dr. Rex L. Smith for their advisory help and for reviewing the manuscript.

Sincere appreciation is extended to the group in Nijmegen including Professor H.F. Linskens for support during my studies, stimulating discussions, and for supplying samples of Petunia pollen. In addition, thanks are given to Dr. M. Kroh for her help with initiating the study of carbohydrates of Petunia pollen eluents and to Dr. M.M.A. Sassen for support and for interesting discussions of pollen morphology and physiology.

I would like to extend my most sincere thanks to Dr. C.A. Hollis of the School of Forest Resources and Conservation for his many suggestions and to Sarah G. Mesa and Joel E. Smith of the Forest Physiology Laboratory for technical assistance. In addition, I gratefully acknowledge Dr. John Gray and the School of Forest Resources and Conservation and the Division of Sponsored Research for support during the course of these studies. Thanks are also due to Dr. Dick Loeppert of the Department of Soil Science for help with liquid chromatography and to Margaret Costanten and Alma Lugo for preparing the figures.

Finally, I must express my humble gratitude to the late Professor R.G. Stanley, under whose supervision these studies were initiated and whose infectous enthusiasm for science must be perpetuated by us all.

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of the University of Florida in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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Edward George Kirby, III

June 1977

Chairman: Indra K. Vasil
Cochairman: Willard W. Payne
Major Department: Botany

Quantitative determinations of components eluted from pollen of 42 angiosperm species representing 25 families and 2 cytologically distinct pollen types were performed. Analyses of the eluents for proteins, carbohydrates, and total elutable substances indicated significant differences between binucleate and trinucleate pollen conditions regarding total elutable compounds as well as elutable carbohydrates and proteins. In all cases binucleate pollen released greater quantities of elutable components than trinucleate pollen.

Analyses of the carbohydrate fraction of pollen eluents of Petunia hybrida L. demonstrated that eluted carbohydrates represent about 20% of the total pollen dry weight. Thin layer chromatography of free sugars in Petunia eluents yielded mannose, glucose, and galactose. In addition, rhamnose, fucose, xylose, arabinose, and glucuronic and galacturonic acids were present in the oligosaccharide fraction.

Although eluted pollen of Petunia possesses poor germination ability in vitro on a simple medium, it produces good pollen tubes on compatible or incompatible styles. Germination of eluted pollen in vitro is significantly restored by the addition of pollen wall eluents to the germination medium. Neutral as well as acidic heat-labile protein components of pollen eluents appear to restore the germination capacity of eluted Petunia pollen. A linear relationship exists between the quantity of eluted protein added and the germination ability of eluted pollen samples.

Polyacrylamide gel electrophoresis and liquid chromatography indicate that the protein component of Petunia pollen eluents contains up to 19 protein components between the range of 5,000 to 61,000 daltons molecular weight. Addition of protein fractions of pollen eluents prepared by ultrafiltration to eluted pollen samples demonstrates that proteins with molecular weights between 50,000 and 100,000 daltons are likely responsible for restoring the germination capacity of eluted pollen.

INTRODUCTION

The significance of mobile fractions of substances within pollen walls has only recently come to the forefront of scientific research. Although considerable knowledge has accumulated regarding allergenic compounds of pollen contributing to hay fever (King and Norman, 1962; Johnson and Marsh, 1966a, b; Underdown and Goodfriend, 1969), limited reports are available concerning the biological and physiological nature of substances that diffuse from pollen grain walls. Protein fractions of pollen wall compounds may be involved as controlling factors in intraspecific incompatibility in poplars (Knox et al., 1972) and interspecific incompatibility in Cosmos bipinnauts (Howlett et al., 1975). Enzymes have been shown to diffuse from pollen grains of many species, and cytochemical studies have demonstrated that these are associated with pollen grain walls (Knox and Heslop-Harrison, 1969, 1970).

The studies presented in this dissertation take several approaches to the study of pollen surface compounds. Angiosperm species have been divided into two distinct groups based on when the division of the generative cell of the pollen grain takes place. In species with trinucleate pollen division of the generative cell occurs prior to shedding; in binucleate pollen generative cell division takes place after shedding. Trinucleate pollen is considered the derived condition (Schnarf, 1939; Brewbaker, 1959; Rudenko, 1959). The cytological condition of pollen at the time of shedding is well correlated with physiological properties, such as germination ability in vitro,

viability during storage, the site of the self-incompatibility reaction and gametophytic or sporophytic control of self-incompatibility (Brewbaker, 1959). Based on studies of 42 angiosperm species representing 25 families, data in Chapter One indicate that the number of pollen nuclei at the time of shedding is also correlated with the quantity of elutable substances, including carbohydrates, proteins and total elutable compounds; greater quantities of all three are present in eluents of species with binucleate pollen.

Further qualitative analyses of the carbohydrate fraction of eluents of Petunia pollen reported in Chapter Two reveal a wealth of monosaccharides and monosaccharide components of oligosaccharides. Elution has very little effect on the germination of Petunia pollen grains on compatible or incompatible styles, however the germination ability of eluted pollen in vitro is significantly reduced. Experimental results show that the germination ability of eluted pollen samples of Petunia may be significantly restored if eluted compounds are added to the germination medium (Chapter Three). Analyses of the eluent fractions reveal that acidic and neutral heat-labile protein fractions of pollen eluents are responsible for boosting the germination of eluted pollen samples. Like the carbohydrate fraction of eluents of Petunia pollen, the protein fraction is also quite diverse, consisting of 19 components separable by electrophoresis and ranging in molecular weight from approximately 5,000 to 61,000 daltons. The germination restoring proteins present in pollen wall eluents seem to be limited to proteins with a molecular weight range of 50,000 to 100,000 daltons.

Through the studies presented here the complex nature of pollen surface compounds becomes apparent, specifically with regard to Petunia,

a species with binucleate pollen and gametophytic control of self-incompatibility. Other available studies of pollen diffusates of Cosmos (Howlett et al., 1975), a species with trinucleate pollen and sporophytic control of self-incompatibility, indicate a less diverse nature of pollen diffusates. The diverse nature of the readily mobile fraction of Petunia pollen walls may be associated with phylogenetically primitive binucleate pollen and gametophytic control of self-incompatibility.

CHAPTER ONE

ELUTABLE SUBSTANCES OF POLLEN GRAIN WALLS*

Introduction

The majority of angiosperm species shed their pollen in the binucleate condition. However, Brewbaker (1967) has shown that in 30% of 2,000 species studied, division of the generative cell takes place prior to shedding. Such pollen grains are shed in the trinucleate condition. It was initially proposed by Schurhoff (1926) and supported by subsequent studies (Schnarf, 1939; Brewbaker, 1959; Rudenko, 1959), that trinucleate pollen is the derived condition and, thus, phylogenetically advanced.

In addition to the cytological difference between binucleate and trinucleate pollen grains, Brewbaker and Majumder (1961) list four distinct characteristics that are strongly correlated with the number of pollen nuclei. Binucleate pollen is usually very easy to germinate in vitro, whereas trinucleate pollen germinates in vitro with considerable difficulty. Binucleate pollen can normally be stored for considerable periods of time under defined conditions, while trinucleate pollen cannot be stored for any appreciable length of time. Intraspecific incompatibility is generally observed in the style or ovary

*This work has appeared in an abbreviated form as: Kirby, E.G. and Smith, J.E.: Elutable substances of pollen grain walls. In "Fertilization in Higher Plants" (ed. H.F. Linskens). North-Holland, Amsterdam. pp. 127-130 (1974).

for species having binucleate pollen, whereas species with trinucleate pollen usually display incompatibility reactions in the stigma. Binucleate species are usually characterized by gametophytic incompatibility systems and trinucleate species are characterized by sporophytic incompatibility systems.

There are notable exceptions, however, to the listed physiological differences. Some well known species have trinucleate pollen that germinates readily in vitro (e.g. Brassica, Pennisetum; Vasil, 1960a, 1962) and in some species having trinucleate pollen the incompatibility reaction takes place in the style (e.g. Beta). In addition, grasses, which have trinucleate pollen, have a well defined gametophytic incompatibility system.

Explanations of the above phenomena are generally based on metabolic requirements for mitosis of the generative cell, which occurs prior to shedding in trinucleate pollen. This mitotic activity apparently deprives the pollen protoplast of substrates essential for germination in vitro, extended storage, and growth of pollen tubes into the style in incompatible matings. Required substrates may be made available to pollen only be genetically compatible stigmas following the action of enzymes or "recognition substances" released from the pollen walls (Knox et al., 1972).

The present work was undertaken to clarify and establish the chemical and physiological characteristics of binucleate and trinucleate pollen in an attempt to further elucidate the biological function of substances contained within the walls of pollen grains.

Materials and Methods

Pollen from 42 species representing 25 families of angiosperms was collected during the 1973-1974 growing season or purchased from the C.G. Blatt and Co., Independence, Missouri, U.S.A. (see Tables 1.1 and 1.2). Pollen was stored at -5°C with silica gel as desiccant.

Samples of 50 mg were extracted for 1 hr at 4°C on a shaker using Coca's solution (Coca, 1922) minus phenol (5.0 g NaCl, 2.75 g $NaHCO_3$ in 1000 ml distilled water).

Aliquots of the extracts were analyzed for proteins by the Lowry procedure (Lowry et al., 1951) and for carbohydrates by α-naphthol-sulfuric acid. In this procedure aliquots of eluents containing not more than 100 µg carbohydrate were added to 5.0 ml α-naphthol reagent (2.0 g α-naphthol in 500 ml conc. H_2SO_4), mixed well and heated in boiling water for 10 min. After cooling optical density readings were taken at 555 nm on a Beckman DB spectrophotometer. The amount of total elutable substances was determined on a dry weight basis. The number of pollen grains in 10 mg samples of each of the 42 species under analysis was determined by evenly suspending 10 mg pollen in 5.0 ml water and counting pollen numbers using a hemacytometer. Calculations of the number of pollen grains per milligram were made accordingly.

Table 1.1

Binucleate Pollen Species Used in Determinations of Carbohydrates (α-Naphthol Procedure), Proteins (Lowry Technique) and Total Elutable Compounds (Dry Weight Basis). All values are expressed as µg per one million pollen grains.

Binucleate Pollen Species	Carbohydrates	Proteins	Total Elutable Compounds
	--------µg/10^6 pollen grains----------		
Betulaceae			
Alnus sp.	10.38	5.20	360.04
Betula papyrifera	9.42	5.06	372.23
Euphorbiaceae			
Ricinus communis	8.84	4.70	389.79
Fagaceae			
Quercus virgiana	8.32	5.17	363.92
Fagus sp.	9.98	5.28	354.96
Juglandaceae			
Juglans nigra	10.13	5.09	370.09
Carya glabra	8.46	4.44	384.91
Leguminosae			
Acacia sp.	9.34	4.90	350.42
Magnoliaceae			
Magnolia grandiflora	9.42	5.04	357.24
Moraceae			
Cannabis sativa	9.43	4.76	378.84
Morus rubra	7.94	4.97	382.42
Myricaceae			
Myrica sp.	9.55	5.37	376.96
Myrtaceae			
Eucalyptus sp.	10.54	4.91	364.11
Oleaceae			
Fraxinus sp.	9.12	4.73	374.44
Olea sp.	10.98	4.89	360.94
Palmae			
Caryota sp.	9.15	5.50	391.84

Table 1.1
(Continued)

Binucleate Pollen Species	Carbohydrates	Proteins	Total Elutable Compounds
	--------μg/10^6 pollen grains----------		
Rosaceae			
Rosa sp.	8.71	4.90	366.92
Pyrus communis	9.21	4.81	364.78
Salicaceae			
Populus sp.	8.62	4.42	376.92
Simarubaceae			
Ailanthus sp.	9.76	5.13	369.41
Urticaceae			
Urtica sp.	10.05	5.31	369.41

Table 1.2

Trinucleate Pollen Species Used in Determinations of Carbohydrates (α-Naphthol Procedure), Proteins (Lowry Technique) and Total Elutable Compounds (Dry Weight Basis). All values are expressed as µg per one million pollen grains.

Trinucleate Pollen Species	Carbohydrates	Proteins	Total Elutable Compounds
	--------µg/10^6 pollen grains----------		
Amaranthaceae			
Amaranthus sp.	6.18	3.38	343.21
Acnida tamariscina	3.98	3.41	319.82
Cactaceae			
Opuntia sp.	6.24	4.29	328.31
Caprifoliaceae			
Sambucus sp.	6.41	4.05	341.19
Chenopodiaceae			
Kochia sp.	6.04	3.94	337.43
Beta vulgaris	5.16	3.67	320.04
Chenopodium album	4.68	3.81	331.17
Compositae			
Chrysanthemum sp.	4.97	3.99	323.23
Artemesia dracunculus	6.58	3.49	334.42
Taraxicum sp.	5.24	3.27	315.01
Helianthus sp.	6.73	4.03	321.11
Baccharis sp.	6.74	4.38	322.81
Chrysothamnus sp.	5.86	3.86	324.56
Ambrosia elatior	5.40	4.20	331.19
Graminae			
Festuca sp.	6.13	4.08	343.21
Zea mays	5.59	3.83	350.13
Plantaginaceae			
Plantago lanceolata	6.62	3.95	317.21
Polygonaceae			
Rumex sp.	5.78	3.64	330.07

Table 1.2
(Continued)

Trinucleate Pollen Species	Carbohydrates	Proteins	Total Elutable Compounds
	--------$\mu g/10^6$ pollen grains----------		
Typhaceae			
Typha sp.	5.61	3.69	327.75
Ulmaceae			
Ulmus americana	4.53	3.72	346.08
Celtis occidentalis	5.38	4.17	339.98

Results

All determinations of proteins, carbohydrates and total elutable compounds have been calculated as micrograms per one million (10^6) pollen grains (Tables 1.1 and 1.2). This represents an effort to minimize any differences related with pollen size. The results show clearly significant differences between binucleate and trinucleate conditions regarding total elutable compounds, as well as elutable carbohydrates and proteins (Table 1.3). This is evident in the greater quantities eluted from species with binucleate pollen.

Table 1.3

Quantitative Differences Between Binucleate and Trinucleate Pollen Conditions

	Binucleate	Trinucleate
	----------µg/10^6 pollen grains----------	
Carbohydrates (α-naphthol)	9.40 ± 1.83**	5.75 ± 1.83
Proteins (Lowry)	4.98 ± 0.57**	3.85 ± 0.57
Total elutable compounds (dry weight basis)	370.97 ± 20.48**	330.15 ± 20.48

Significance:
** - 0.01

Discussion

Tsinger and Petrovskaya-Baranova (1961) concluded from the presence of proteins and other compounds that pollen grain walls are ". . . living, physiologically active structures playing a very responsible role in the processes of interchange between pollen grain and its substrate" (p. 106). Studies of Mäkinen and Brewbaker (1967) and Stanley and Search (1971) showed that proteins and enzymes contained within pollen walls can be eluted within 5 sec, indicating a rapid interchange between pollen grain and substrate.

Cytochemical studies (Knox and Heslop-Harrison, 1969, 1970; Vithanage and Knox, 1976) have shown that enzymes are associated with the intine layer of the pollen wall. More recently, attention has focussed on gametophytic and sporophytic fractions of pollen wall compounds. The difference between these two fractions is the site of their synthesis. Heslop-Harrison et al. (1973), Howlett et al. (1975) and Vithanage and Knox (1976) have reported that protein fractions as well as allergenic and antigenic fractions may be associated with exine and intine sites. Their observations indicate that intine-held compounds are gametophytically synthesized, while compounds located in the exine are sporophytically synthesized. Furthermore, it has been proposed that control of gametophytic intraspecific incompatibility is mediated through intine-held recognition substances, while exine-held compounds control sporophtyic incompatibility systems (Heslop-Harrison et al., 1973).

The results of this preliminary study indicate that cytologically distinct groups of angiosperm pollen (binucleate and trinucleate) that

have been reported to differ in several physiological aspects, including type of incompatibility control, also display quantitative differences in elutable components that have been both gametophytically and sporophytically contributed. Values reported in Tables 1.1, 1.2 and 1.3 for total elutable substances, calculated on a dry weight basis, are considerably higher than values reported for elutable carbohydrates and proteins. Components, including flavinoids, organic acids, minerals and others, are likely to form the bulk of the elutable substances. If, indeed, control of incompatibility is regulated through "recognition substances" carried within the walls of pollen grains, then qualitative studies characterizing distinct fractions of these compounds could elucidate possible control mechanisms involved in intraspecific incompatibility.

CHAPTER TWO

POLLEN WALL ELUENTS OF *Petunia hybrida* L. THE CARBOHYDRATE COMPONENT

Introduction

Recently, considerable attention has focussed on wall components of pollen grains, specifically with regard to possible functions of readily mobile fractions in cell recognition and incompatibility on the stigma, and pollinosis in man. Although the structure of pollen grain walls has been studied in detail (for reviews see Heslop-Harrison, 1968a; Vasil, 1973; Stanley and Linskens, 1974), few recent studies have focussed on their chemical constituents, with the exception of work on allergens (King and Norman, 1962; King *et al.*, 1964; Johnson and Marsh, 1966a, b; King *et al.*, 1967; Underdown and Goodfriend, 1969; Griffiths and Brunet, 1971). The outer layer of the pollen wall, the exine, is composed of oxidized carotenoid derivatives (Brooks and Shaw, 1968). The wall layer closest to the haploid pollen protoplast, the intine layer, is generally thought to be cellulosic in nature (Sitte, 1953; Heslop-Harrison, 1968b), but also contains non-cellulosic pectinaseous materials (Roland, 1971; Knight *et al.*, 1972). The complex sporopollenin component is derived from both microspore protoplast and from polyploid or multinucleate tapetal cells of the anther sac (Heslop-Harrison, 1971; Vasil, 1973).

In addition to structural components of pollen walls, a rapidly diffusing fraction has been demonstrated in many species, as is shown

by the work of several groups. However, the major emphasis of the majority of this work has been centered on protein components of pollen diffusates. With the exception of the work of Howlett *et al*. (1975), who found the major carbohydrate component of pollen diffusates of *Cosmos bipinnatus* to be arabinose, little analytical work has focussed on the diffusible carbohydrates of pollen walls. Because of the sizeable carbohydrate fraction found in eluents from pollen of 42 angiosperm species (Chapter One), the present study was undertaken to further characterize the carbohydrate fraction of readily mobile pollen wall compounds.

Materials and Methods

Pollen Collection. Pollen of Petunia hybrida, clone W166K with self-incompatibility alleles S_1S_2 was obtained from plants grown in the green house. Anthers were removed prior to anthesis and allowed to open in Petri plates. Pollen was then collected, separated from extraneous material by the use of sieves and was used fresh or after storage at -5°C over silica gel.

Preparation of Eluents. To obtain eluents, 50 mg samples of pollen were treated in the following four ways: (1) The pollen sample was placed on a stainless steel sieve (5 µm mesh) and suspended in 10 ml 1% NaCl, pH 7.5 (Knox et al., 1972). Vacuum was applied from a water aspirator and the extract filtered through the sieve and collected. No pollen was present in the eluents. Total elution time was 1 min. (2) The pollen sample was placed on a stainless steel sieve (5 µm mesh), suspended in 20 ml 1% NaCl, pH 7.5, and the same procedure followed as in (1) above. Elution time was 2 min. (3) The pollen sample was placed in an Erlenmeyer flask with 10 ml 1% NaCl, pH 7.5, and shaken for 30 min at 4°C. Pollen was separated from the eluent by centrifugation at 500 x g for 10 min. (4) The pollen sample was suspended in 3.0 ml 1% NaCl, pH 7.5, and homogenized using a MSE 150 Watt ultrasonic disintegrator for a total of 5 min in 30 sec intervals. Microscopic examination revealed that nearly all pollen grains were broken after this treatment. The homogenate was centrifuged at 10,000 x g for 30 min and the supernatant decanted. Pollen eluents and the supernatant of (4) were evaporated to dryness on a flash evaporator and

taken up in 1 ml cold double distilled water. All above operations were performed in the cold to minimize breakdown of eluted compounds by enzymes present in pollen eluents.

Analytical Procedures

Separation of Neutral and Acidic Sugars. Immediately after preparation eluents and the supernatant of (4) were passed through Dowex-50 (H+) short columns, evaporated to dryness and solubilized in 1 ml double distilled water. Free neutral and acidic sugar fractions of Dowex-50 effluents were separated on a Dowex-1-formate column according to the procedure of Kroh (1973) and aliquots were chromatographed directly. Oligosaccharide and components of Dowex-50 effluents were hydrolyzed with 2 N trifluoracetic acid (TFA) for 1 hr at 121°C, or with 80% TFA (v/v) for 48 hrs at 121°C prior to separation of neutral and acidic sugar fractions. TFA hydrolysates were evaporated and acid removed by repeated addition of water and evaporation. Neutral and acidic sugar components of hydrolysates were separated by Dowex-1-formate columns and aliquots of Dowex-1 effluents were chromatographed.

Chromatography. Thin layer chromatographs of neutral sugars were developed in ethyl acetate-pyridine-water (80:20:10) (v/v). Chromatographs of acidic sugars were run in acetone-n-butanol-0.1 M NaH_2PO_4 (40:25:35) (v/v). Standards utilized were galactose, glucose, mannose, arabinose, xylose, fucose, and rhamnose as neutral sugars and galacturonic and glucuronic acids as acid sugars.

Sugars were detected on chromatographs with methanolic $AgNO_3$, KOH and sodium thiosulfate reagents.

Total Carbohydrate Determination. Qunatitative determination of total carbohydrates was performed according to the phenol-sulfuric acid method of DuBois et al. (1956).

Electron Microscopy. Fresh and eluted pollen grains of Petunia hybrida were fixed in 3.0% glutaraldehyde prepared in 0.1 N phosphate buffer, pH 7.0, for 30 min to 1 hr, washed with two changes of buffer and post fixed for 3 hrs in 3.0% OsO_4 or 3.0% $KMnO_4$ prepared in the same buffer. All fixations were at room temperature. Post-fixation was followed by buffer and distilled water washes and agar embedding. Dehydration was carried out in an ethanol series followed by propylene oxide and pollen grains were embedded in Epon.

Thin sections of pollen grains were cut on a Porter-Blum MT-2 ultramicrotome using a diamond knife. Sections were post-stained with saturated aqueous uranyl acetate followed by Reynolds' (1963) lead citrate. Electron micrographs were made using a Philips EM 300 electron microscope operating at 60 kV.

Pollen Germination Assays

In Vitro Assay. Fresh or eluted pollen grains of Petunia hybrida were germinated in 10% sucrose, 100 ppm boron (supplied as H_3BO_3) for 3 hrs at 30°C. The concentration of pollen was maintained at 5 mg/ml germination medium. Pollen grains were considered germinated after 3 hrs if pollen tubes were extended to a length equal to or greater than the diameter of the pollen grain.

In Vivo Assay. Fresh or eluted pollen was applied to compatible styles of Petunia hybrida (clone T_2U). The pollinated styles plus

pedicels were first put in 2 ml water in small vials and placed in a light box or growth chamber at 27°C. Pollen tube length was measured after 24 hrs by staining callose in pollen tubes in stylar squash mounts with aniline blue (0.005% in 0.15 M KH_2PO_4, pH 8.2) (Jensen, 1962) and observing under a Leitz ultraviolet microscope.

Results

Carbohydrate Content of Petunia Pollen Eluents. Total carbohydrate determinations of pollen eluents and the supernatant of homogenized pollen are shown in Table 2.1. Carbohydrates are very quickly eluted from Petunia pollen in quantities that approach 20% of the total dry weight of the pollen sample (Fig. 2.1).

Qualitative Analysis of Elutable Substances by Thin Layer Chromatography. Separation by thin layer chromatography (TLC) of free sugars as well as hydrolysis products of the pollen eluents and the supernatant of homogenized polled are presented in Table 2.2. The composition of free sugars of (1) and (2) is identical: both contain mannose, glucose and galactose. These sugars are also present as free neutral sugars in (3) and (4). Hydrolysis of the oligosaccharide components of (1) yields rhamnose, fucose and xylose in addition to mannose, glucose and galactose. In (2) arabinose was also found as an hydrolysis product. Hydrolysis of oligosaccharide fractions yielded glucuronic and galacturonic acids from both (1) and (2). In all chromatograms of Dowex-1-formate column effluents developed in acetone-n-butanol-0.1 M NaH_2PO_4 (40:25:35) for the separation of acid sugars an additional spot, which did not co-chromatograph with galacturonic or glucuronic acid standards, was detected with alkaline silver nitrate. This spot may represent an unknown uronic acid, sugar phosphate, aldonic acid or other compound.

Aliquots from (3) and (4) contained identical free neutral and acidic sugar fractions before and after hydrolysis (Table 2.2). Mannose,

Table 2.1

Carbohydrate Contents of Eluents of Pollen of *Petunia hybrida*

	mg Carbohydrate Per 50 mg Pollen	Per cent of Total
(1) (1 min elution)	7.20	60.0
(2) (2 min elution)	9.36	78.0
(3) (10 min elution)	10.0	83.3
(4) (disrupted pollen)	12.0	100

Eluents from (1), (2), (3), and (4) refer to numbers used in the text for 10 ml, 20 ml, shaken and eluents of disrupted pollen, respectively. Carbohydrate determinations were performed according to the technique of DuBois *et al*. (1956).

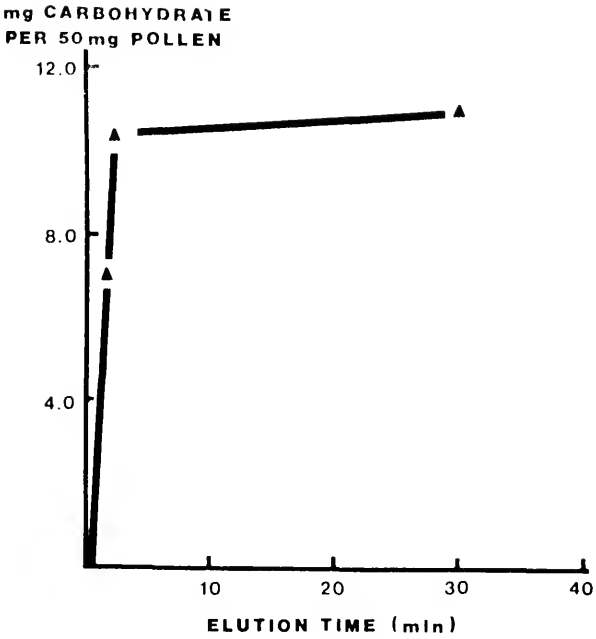

Figure 2.1. Effect of elution time on quantity of carbohydrate eluted from pollen of Petunia hybrida. Carbohydrate determinations were performed according to the technique of DuBois et al. (1956).

Table 2.2

Chromatographic Analyses of Carbohydrate Fractions of Eluents of Petunia Pollen

	(1)		(2)		(3)		(4)	
	free	TFA hydrolysate	free	TFA hydrolysate	free	TFA hydrolysate	free	TFA hydrolysate
Neutral sugars								
rha	-	+	-	+	-	+	-	+
fuc	-	+	-	+	-	+	-	+
xyl	-	+	-	+	-	+	-	+
ara	-	-	-	+	-	+	-	+
man	+	+	+	+	+	+	+	+
glu	+	+	+	+	+	+	+	+
gal	+	+	+	+	+	+	+	+
Uronic acids								
glu UA	-	+	-	+	+	+	+	+
gal UA	-	+	-	+	+	+	+	+

Eluents (1), (2), (3), and (4) refer to numbers used in the text for 10 ml, 20 ml, shaken and eluents of disrupted pollen, respectively. Abbreviations employed: rha, rhamnose; fuc, fucose; xyl, xylose; ara, arabinose; man, mannose; glu, glucose; gal, galactose; glu UA, glucuronic acid; gal UA, galacturonic acid. A minus (-) indicates that the monosaccharide was not present on chromatograms; a plus (+) indicates that the monosaccharide was detected. Chromatograms of neutral sugars were run in ethyl acetate-pyridine-water (80:20:10) (v/v); acid sugars were run in acetone-n-butanol -- 0.1 M NaH_2PO_4 (40:25:35) (v/v).

glucose and galactose were present as free neutral monosaccharides, and glucuronic and galacturonic acids were present as free uronic acids. These acid sugars were not observed in the free sugar fractions of (1) and (2). Hydrolysis of the oligosaccharide fraction of (3) and (4) also yielded identical monosaccharide components, rhamnose, fucose, xylose, arabinose, mannose, glucose, and galactose as well as glucuronic and galacturonic acids (Table 2.2).

Since the solvent system employed for chromatography of neutral sugars (ethyl acetate-pyridine-water) cannot unequivocally resolve fucose and xylose, some question may be raised as to whether both monosaccharides are present in hydrolysates of oligosaccharide fractions of pollen eluents. However, in all chromatograms of hydrolysates, two spots were clearly distinguished which co-chromatographed with xylose and fucose of standard sugar mixtures.

Electron Microscopy. Electron micrographs of eluted and non-eluted pollen grains of Petunia hybrida display similar cytoplasmic detail (Figures 2.2 and 2.3). Plasma membranes of eluted material appear undisturbed by the elution procedure. No bursting was observed. In addition no basic difference was observed in the ultrastructural appearance of intine and exine layers of the pollen walls of eluted and non-eluted samples. However, the arrows in Figure 2.2 point to empty areas between the intine and the plasmalemma. Such areas were frequently observed in sections from eluted pollen and occasionally seen in sections from non-eluted pollen. These areas may represent sites of eluted intine-held fractions, however, their occurrence being a result of fixation procedures is not precluded.

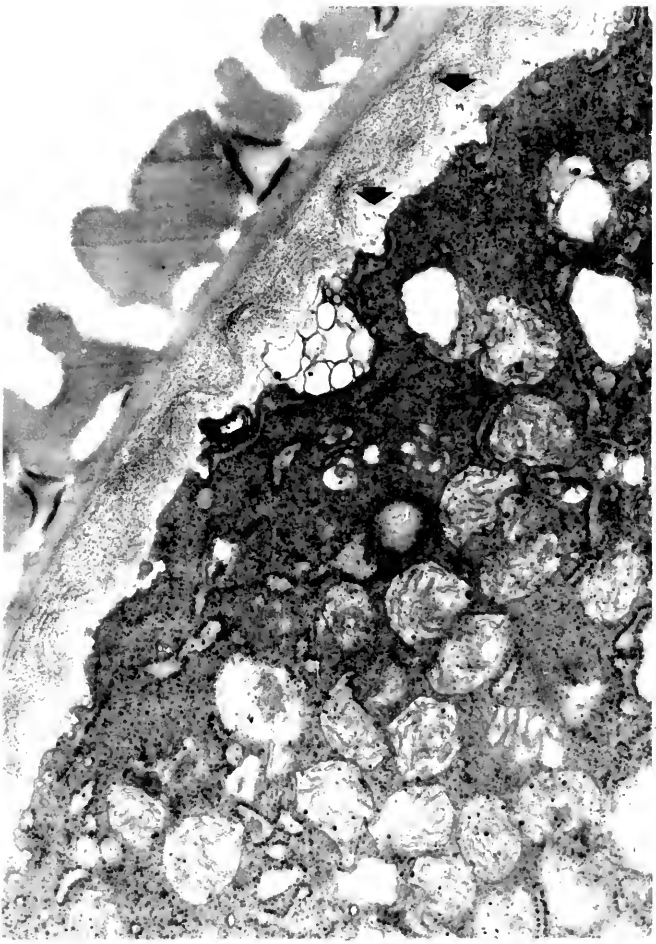

Figure 2.2. Pollen grain of Petunia hybrida after elution with 10 ml 1% NaCl, pH 7.5. Cytoplasmic detail is similar to that of non-eluted pollen; the plasmalemma is continuous. Spaces (arrows) within the intine layer are frequently observed. (Magnification 19,200 x)

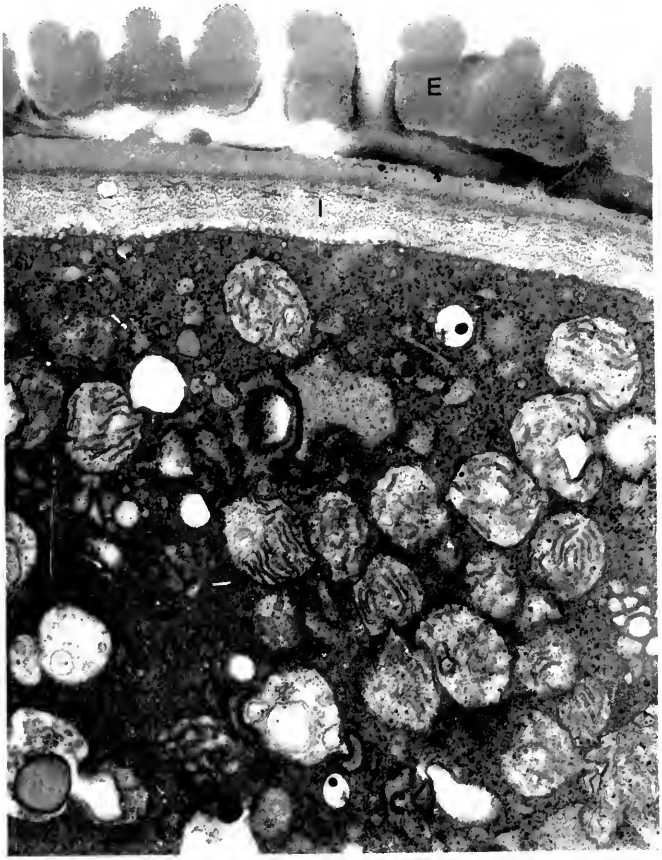

Figure 2.3. Fresh, non-eluted pollen grain of Petunia hybrida. Exine (E) and intine (I) layers are clearly seen, as well as typical cytoplasmic organelles. (Magnification 19,200 x)

Table 2.3

Pollen Germination Tests of Eluted Pollen Fractions

Pollen Sample	Per cent of Control
(1) (1 min elution)	29.3%
(2) (2 min elution)	4.9%

(1) represents values for pollen eluted with 10 ml 1% saline, pH 7.5.

(2) represents values for pollen eluted with 20 ml 1% saline, pH 7.5.

Values are reported as percentages of control germinations of non-eluted pollen samples. Values are means of 4 replications of two experiments. For each replication 400 pollen grains were counted.

Table 2.4

Pollen Tube Length in Compatible Pollinations with Eluted Pollen Grains of Petunia

Cross	Mean Tube Length (mm)	
T_2H x W166K	20.7	s = 1.89
T_2H x W166K (1)	20.7	s = 2.87
T_2H x W166K (2)	16.4	s = 7.07

Results are reported for female plants (T_2H) crossed with pollen (W166K) eluted with 10 ml saline, pH 7.5 (1); pollen eluted with 20 ml saline, pH 7.5 (2); and non-eluted control pollen. Numbers (T_2H and W166K) refer to clones of Petunia hybrida. Values for standard deviations (s) are also given. All pollinations were performed in quadruplicate.

Germination Tests. In vitro germination tests showed that eluted pollen germinates poorly (Table 2.3). The values range from 4.9% for pollen eluted with 20 ml saline solution to 29.3% for pollen eluted with 10 ml saline. Values are expressed as per cents of control germinations. The low values reported for germination of eluted pollen may be caused by the effect of saline during the elution process, and/or by the removal of wall-bound substances required for germination. However, in vivo germination assays of eluted pollen samples performed on compatible styles showed good pollen tube growth (Table 2.4). Pollen tube length of samples eluted with 10 and 20 ml saline solution (20.7 and 16.14 mm respectively) compares favorably with values for non-eluted controls (20.7 mm).

Discussion

From this study it is concluded that a wealth of carbohydrate material is present in eluents of pollen grains of Petunia hybrida. However, the origin of this diverse carbohydrate fraction remains a mystery.

One may assume that the method and duration of elution determines if substances present in eluents are derived from wall sites, or are also contributed by haploid pollen protoplasts. It appears likely that quantitatively less carbohydrate material is removed from the pollen cytoplasm when pollen is eluted for 1 or 2 min, rather than shaken for 30 min. Both quantitative and qualitative sugar determinations support this. Eluents prepared by washing pollen with 10 ml and 20 ml saline solution contain monnose, glucose and galactose as free sugars. Eluents prepared by shaking for 30 min and the supernatants of homogenized pollen contain glucuronic and galacturonic acids in addition to the above monosaccharides as free sugars. Since only supernatants of homogenized pollen and 30 min eluents contain glucuronic and galacturonic acids as free sugars, it is probable that these sugars and likely other components found in 30 min eluents may be of cytoplasmic origin. Ten and 20 ml eluents do not contain free glucuronic and galacturonic acids which may indicate that cytoplasmic components are not present. In all cases hydrolysates of oligosaccharide fractions contained rhamnose, fucose, xylose, mannose, glucose, galactose, glucuronic acid, and galacturonic acid. Arabinose was present in all hydrolysates, excepting the 10 ml eluents. Electron micrographs of eluted pollen compared with

those of non-eluted pollen grains reveal no difference in morphological appearance of the cytoplasm after elution. The plasma membranes of eluted pollen grains appear undisturbed. This leads to further support of the contention that cytoplasmic constituents are not present in 10 ml eluents.

The observation that eluted pollen is able to germinate in compatible pollinations also supports the contention that protoplasmic integrity is not altered during elution. This has also been shown to be the case with eluted pollen of Lilium longiflorum (Fett et al., 1976), another example of gametophytic self-incompatibility. However, eluted pollen grains of Petunia germinate poorly in vitro, while the removal of loosely bound substances from lily pollen resulted in little decrease in germination ability. In Petunia the decrease in pollen germination ability may be due, in part, to effects of the eluting solution (saline), or may be caused by the removal of specific factors from wall sites (Chapter Three). Wall-held compounds may function as a source of enzymes or nutrients required for pollen tube elongation. Such factors could be supplied by female tissues to eluted pollen grains during germination on compatible stigmas.

Monosaccharides present in eluents of Petunia pollen are commonly found as components of higher plant cell walls (Lamport, 1970). Hydrolysates of pollen wall diffusates from Cosmos bipinnatus (Howlett et al., 1975) contained only arabinose and possibly glucose and galactose. Glucuronic and galacturonic acids could not be detected. However, procedures employed by Howlett's group eliminated compounds with molecular weights less than 10,000 daltons. Eluents of Petunia pollen contain diverse carbohydrates, including free sugars and

oligosaccharides. It is quite possible that such diversity also is present in the carbohydrate fraction of eluents of pollen of Cosmos bipinnatus, but did not appear in analyses of wall diffusates.

Studies on homogenized rose pollen (Zolotovitch and Secenska, 1962 and Zolotovitch et al., 1964) have demonstrated that glucose, galactose and rhamnose are present. Knight et al. (1972) have shown that uronic acids, as determined by the microdecarboxylation method, are major components of pollen of 53 angiosperm and 5 gymnosperm species. This uronic acid fraction is likely the pectinaceous intine matrix material reported by Roland (1971). Pectin fractions of intine materials probably function in the initial uptake of water by germinating pollen grains, since pectins influence water distribution within plant cell walls (Northcote, 1972). Galacturonorhamnans containing an α-D-galacturonic acid-L-rhamnose backbone with side chains of L-fucose, D-xylose and D-galactose are the major constiuents of plant pectins (Northcote, 1972). These monosaccharides are found as hydrolysis products of wall eluents of Petunia pollen and it is conceivable that elutable oligosaccharide fractions of wall eluents containing such sugars are soluble precursors of pectinaceous material of the intine.

Intercellular material from stylar transmitting tissue of Petunia hybrida contains a mixture of low molecular weight carbohydrates (Kroh, 1973). This material provides nutrients for growing pollen tubes. Analysis of the carbohydrate component of eluents of pollen of Petunia hybrida revealed a mixture of acidic and netural monosaccharides as components of the oligosaccharide fraction. In addition free neutral and acidic sugars are present. If the function of the

carbohydrate fraction of pollen eluents is similar to the function of the carbohydrate fraction of intercellular stylar transmitting tissue, we may expect some degree of homology of composition.

CHAPTER THREE

EFFECT OF POLLEN ELUENTS AND POLLEN ELUENT FRACTIONS ON GERMINATION OF ELUTED POLLEN SAMPLES OF *Petunia hybrida* IN VITRO

Introduction

Much of the early work on protein components of pollen has come from apiarists, since pollen functions as the major source of amino acids for bees, and from allergists, since the causal factors in pollen allergies are thought to be proteins. Pollen has been shown to contain from 7.0% protein (lodgepole pine) to 35.1% protein (date palm) (Todd and Bretherick, 1942). Hydrolysates of pollen proteins contain all amino acids commonly found in plant tissues (Auclair and Jamieson, 1948). Studies on pollen allergens have mainly focussed on rye grass and ragweed species. Allergenic proteins are generally low molecular weight compounds (5,000-30,000 daltons) and contain carbohydrate moieties (Abramson, 1947; Marsh *et al.*, 1966).

Enzymes have been observed to diffuse from pollen, as determined by starch liquification, and a function for diffusable enzymes in pollen tube nutrition was postulated early (Green, 1894). More recently, Haeckel (1951) studied amylase, phosphatase and invertase activities in pollen of 20 angiosperm species and found phosphatase activities to be the highest prior to germination. Russian workers (Poddubnaya-Arnoldi *et al.*, 1959) described the release of many enzymes by angiosperm pollen and traced the localization of these enzymes to the pollen

grain wall. Tsinger and Petrovskaya-Baranova (1961) determined that additional enzymes are present in pollen walls of peony and Amaryllis, including dehydrogenases, cytochrome oxidase and acid phosphatase. These workers concluded that, because of its wealth of typical cytoplasmic enzymes, the pollen wall must be termed a living physiological structure.

Mäkinen and Lewis (1962) demonstrated that antigen-reacting enzymes diffuse from the surfaces of pollen of Oenothera when placed on moist agar. The pattern of diffusion of proteins from germinating Petunia pollen has been described by Stanley and Linskens (1964, 1965). These workers also determined that sucrose metabolizing activity is localized on the surface of Petunia pollen and diffuses rapidly into the growth medium. Lewis et al. (1967) determined that washing pollen grains of Oenothera with an aqueous, non-germination promoting medium releases catalase, amylase, esterase, phosphatase and leucine aminopeptidase activities. Furthermore, Stanley and Thomas (1967) reported the release of high amounts of cellulase from pollen of cattail, pine and pear prior to germination. It appears that pollen grains of many angiosperm species are actively involved in releasing proteins, especially enzymes, just prior to germination.

Recent cytochemical studies have revealed that protein components are found in the walls of pollen grains, both in the pectocellulosic intine, particularly in pore regions, and in sexine cavities of the exine. These components include readily diffusible allergenic and enzyme fractions (Knox and Heslop-Harrison, 1969, 1970, 1971a; Heslop-Harrison et al., 1973; Vithanage and Knox, 1976). Furthermore, it has been shown that antigenic proteins found in the exine layer of pollen

grains of Iberis (Cruciferae) are lost to the stigma within 5 to 10 minutes following compatible or incompatible pollinations (Heslop-Harrison et al., 1974). It has also been suggested that mobile pollen wall proteins are directly involved in incompatibility reactions (Knox et al., 1970; Knox and Heslop-Harrison, 1971a, b; Knox, 1971; Knox et al., 1972; Howlett et al., 1975). However, the work of Fett et al. (1976) indicates that mobile proteins are not involved in the self-incompatibility response in Lilium longiflorum.

The previous chapter described the complexity and possible functions of the carbohydrate components of eluents of Petunia pollen walls. The present chapter reports the stimulatory effect of proteinaceous fractions of wall eluents of Petunia pollen on germination of eluted pollen grains in vitro. In addition consideration is focussed on chemical properties of the stimulatory fraction and its mode of action.

Materials and Methods

Pollen Collection and Storage. Pollen of Petunia hybrida, clone W166K, was collected and stored as described previously (Chapter Two).

Preparation of Eluents. Samples of pollen (25 mg) were placed on a Millipore filter (0.4 μm pore diameter) mounted on a filter support funnel. Pollen was suspended in 20 ml double distilled water, the water drawn through the filter by vacuum and the resulting eluent collected in a vacuum flask. The elution process was performed in an ice water bath and using ice cold solutions. Eluted pollen grains remained on the Millipore filter and were easily transferred to incubation flasks for germination tests.

Water was removed from eluent samples using a flash evaporator. Eluents prepared in this manner were used fresh in further analyses and germination assays, or stored in the freezing compartment of a refrigerator at -5°C.

Separation of Neutral, Basic, Acidic and Non-Polar Fractions of Eluents. Eluents from 100 mg pollen (4 x 25 mg samples) were redissolved in 20 ml double distilled water, pH adjusted to 3.0 with 0.5 N HCl, and partitioned against re-distilled ethyl acetate (Hollis and Tepper, 1971). Under these conditions basic compounds are freely soluble in the aqueous, pH 3.0 phase. The remaining ethyl acetate phase was partitioned against water at pH 8.5 (adjusted with 0.4 N NH_4OH). The resulting aqueous phase contained freely soluble acidic compounds. The remaining ethyl acetate was lastly partitioned against double distilled water (pH 6.0). The aqueous phase contained freely soluble neutral compounds,

while non-polar compounds were contained within the ethyl acetate phase. The four fractions thus obtained (pH 3.0, 6.0, 8.5 and ethyl acetate) were dried using a flash evaporator. Residues from the pH 3.0 and pH 8.5 phases were washed with double distilled water and repeatedly evaporated to remove all traces of HCl or NH_3. All operations were carried out in the cold. The four dry fractions were used directly to test their effects on germination of eluted pollen, or used in further preparative procedures.

Separation of Carbohydrates and Amino Acid-Protein Fractions. Aliquots of pH 6.0 and pH 8.5 phases obtained by partitioning procedures were placed on a short Dowex 50W-8X cation exchange column and eluted with 0.4 N NH_4OH, yielding the carbohydrate fraction, followed by elution with 4.0 N NH_4OH, yielding the amino acid-protein fraction. All Dowex effluents were dried in a flash evaporator, repeatedly washed with double distilled water and evaporated to remove all traces of NH_3. The amino acid-protein and carbohydrate fractions were then redissolved in pollen germination medium and their effects on the germination of eluted pollen grains assayed.

Denaturation. Aliquots of eluents of Petunia pollen were sealed in hydrolysis tubes and placed in an autoclave at 121°C for 90 min. This procedure causes proteins to lose their tertiary structural characteristics.

Preparation of Acetone Protein Precipitates. Proteins in eluents of Petunia pollen were precipitated according to a modification of Hare's (1970) procedure. Dry eluents were taken up in 70% acetone and the creamy suspension centrifuged at 27,000 x g for 20 min. The resulting pellet was washed two times with 70% acetone followed by two

washes with 100% acetone. All procedures were performed in the cold. The final white precipitate was dried with an air stream. This material is referred to as the acetone precipitated protein fraction.

The dried precipitate was dissolved in pollen germination medium or in protein extracting solution modified from Hare (1970) as follows:

Urea	15 g
$K_2S_2O_5$	0.5 g
Ascorbic Acid	1.0 g
Cleland's Reagent	0.1 g
Tween 20 (10% solution)	4.0 ml
Tris to pH 8.5	
Double distilled water to 100 ml	

Forty microliters of the extractive, which contained about 45 µg protein, were applied to precharged polyacrylamide gels and developed at 2.5 milliamperes per gel. Tris-glycine buffer (pH 8.5) containing 0.05% bromophenol blue was in the upper chamber and tris-HCl buffer (pH 8.1) was in the lower chamber. After 70 min gels were removed and shaken in 10% aqueous trichloroacetic acid (TCA) for 30 min followed by staining for 48 hrs with 0.25% Coomassie blue added to fresh TCA.

Molecular Weight Determination. The range of molecular weights of proteins of eluents of Petunia pollen was determined using a Waters AGL/GPC 502 liquid chromatograph equipped with a model 6000 solvent delivery system and using a 1.5 m Porasil CX (Waters Associates) molecular sieve column. Water was used as solvent. All operations were performed at room temperature. Proteins in effluents were detected

with a UV flow detector and the flow rate was 0.2 ml per min. Column operating efficiency was determined to be 1680 theoretical plates.

Molecular weight ranges were calculated relative to a mixture of known molecular weight protein standards.

Total Protein Precipitation and Protein Fractionation as to Molecular Weight. Lyophilized eluents from 250 mg pollen were taken up in 20 ml ice cold water and placed in a 50 ml flask on ice. Ammonium sulfate (7.2 g) was added and the mixture stirred on ice for 15 min. The resulting suspension was centrifuged 20 min at 28,000 x g in a refrigerated contrifuge. The supernatant was decanted and the protein pellet taken up in 5 ml cold distilled water, transferred to a dialysis bag and dialyzed against water overnight to remove all traces of ammonium sulfate. The resulting dialysate was used directly for further fractionation, or frozen at -20°C for later use.

Total protein samples prepared in this manner were taken up in 100 ml cold distilled water and placed on an Amicon High Performance Thin Channel Diafiltration System equipped with an LP-1 pump. This system can be used to separate proteins as to approximate molecular weight and the procedures for fractionating proteins of Petunia pollen eluents is diagrammed in Figure 3.1.

Carbohydrate Assay. Total carbohydrates present in protein fractions of pollen eluents were determined by the phenol-sulfuric acid technique of DuBois et al. (1956).

Protein Assay. All protein determinations were performed according to the procedure outlined by Lowry et al. (1951).

In Vitro Pollen Germination. Germination tests of eluted and non-eluted pollen samples of Petunia hybrida were performed in 0.05 M

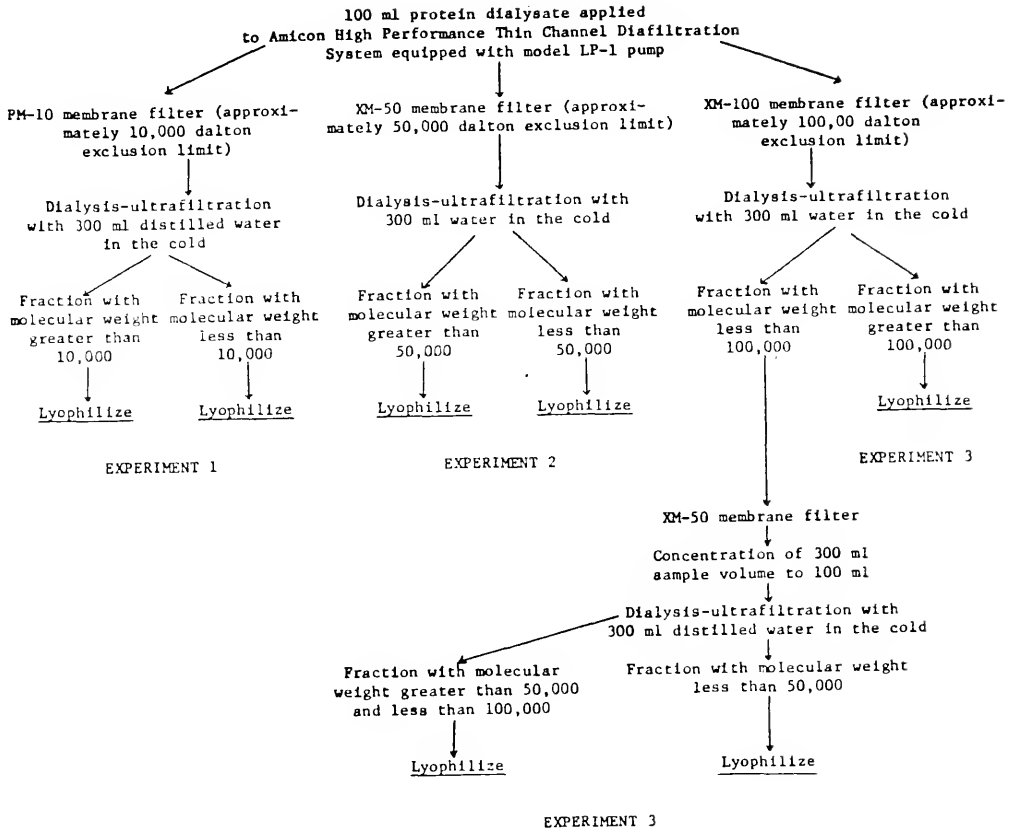

Figure 3.1. Ultrafiltration procedure for the fractionation of dialyzed ammonium sulfate precipitated proteins from eluents of pollen of Petunia hybrida.

calcium phosphate buffer (pH 6.0) containing 0.4 M raffinose and 80 ppm boron, supplied as H_3BO_3. Eluents and protein fractions were dissolved in germination medium prior to the addition of pollen. Germination was at 27°C for 3 hrs on a water bath shaker and pollen concentration was maintained at 5 mg per ml (25 mg per 25 ml).

Results

The effect of duration of elution on the quantity of protein eluted, as determined by the Lowry procedure, is shown in Figure 3.2. The data show that 2 min elutions release 84.2% of the total protein eluted in 10 min and are likely to contain almost all of the sporophytic components of pollen wall eluents, but also much of the gametophytic protein components of the pollen wall.

Table 3.1 presents the results of a germination test involving non-eluted control pollen of Petunia hybrida and pollen after elution. Such tests indicate that elution has greatly reduced the ability of pollen to germinate. After three hours no noticible difference was observed in pollen tube length between eluted and control samples. Throughout all experiments pollen tube length averaged 97.6 μ after 3 hrs. The percentage of pollen grains that germinated varied somewhat with the pollen sample used. If eluted compounds are added to eluted pollen samples in amounts present in non-eluted controls, the germination capacity of eluted pollen is significantly restored (Table 3.1). Non-eluted control pollen germinated at approximately 51-64%, the variation being due to the pollen sample.

Results of adding acidic, basic, neutral and non-polar fractions of pollen eluents to germinating eluted pollen are reported in Table 3.2. The neutral fraction (pH 6.0) and the acidic fraction (pH 8.5) considerably increase per cent germination of eluted pollen (83.4% and 101.4%, respectively). The acidic fraction boosts the per cent germination of eluted pollen to the level of non-eluted controls. Addition of

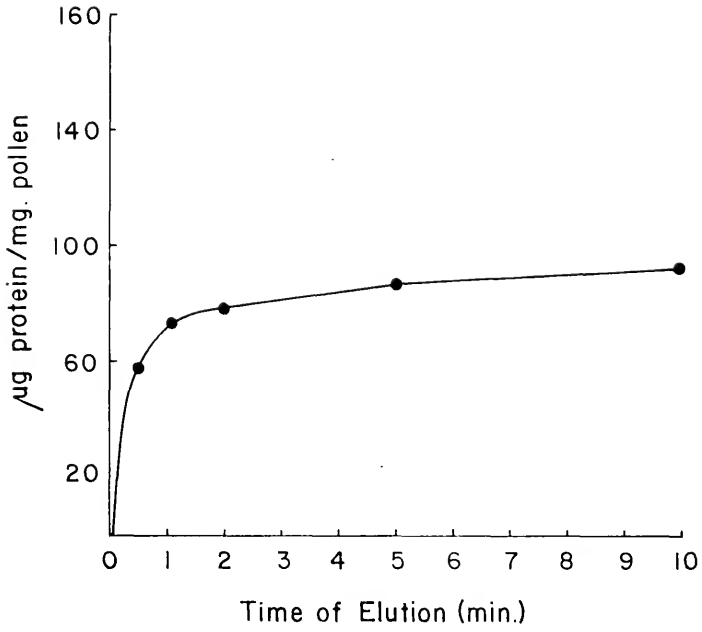

Figure 3.2. Effect of time of elution on quantity of protein eluted from pollen of Petunia hybrida. Proteins were determined according to the Lowry procedure (Lowry et al., 1951).

Table 3.1

The Effect of Pollen Eluents of *Petunia hybrida* on Germination of Eluted Pollen Samples

Treatment	Germination (Per cent of Control)
Control	100
Eluted pollen (20 ml water for 2 min)	48.8
Eluted pollen plus eluent	71.8

Values are reported as per cents of control germinations, which ranged from approximately 51% to 64%, the variability being dependent on the pollen sample. Total eluents were prepared as described in the text. All germinations involved 25 mg pollen; in all cases eluent added is that quantity derived from 25 mg pollen.

Table 3.2

The Effect of Basic, Neutral, Acidic, and Non-Polar Fractions of Eluents of *Petunia hybrida* Pollen, Prepared by Ethyl Acetate Partitioning, on Germination of Eluted Pollen Samples

Treatment	Germination (Per cent of Control)
Control	100
Eluted Pollen	50.5
pH 3.0 phase (basic fraction)	4.6
pH 6.0 phase (neutral fraction)	83.4
pH 8.5 phase (acidic fraction)	101.4
ethyl acetate phase (non-polar fraction)	14.9

Values reported are per cents of control germinations. All germinations involved 25 mg pollen; in all cases eluent added is that quantity derived from 25 mg pollen. Values are means of 4 replications of three experiments. For each replication 400 pollen grains were counted.

basic and non-polar fractions decreases the germination capacity of eluted pollen.

Table 3.3 presents the effects of amino acid-protein and carbohydrate components of neutral and acidic fractions of pollen wall eluents on per cent germination of eluted pollen grains. Protein-amino acid components eluted from Dowex 50W-8X columns with 4.0 N NH_4OH of both acidic and neutral fractions are effective at boosting the per cent germination of eluted pollen close to that of controls (97.4% and 80.9%, respectively). When carbohydrate components of acidic and neutral fractions eluted from Dowex 50W-8X columns are added, no stimulation of pollen germination was observed (Table 3.3).

Table 3.4 shows that after heat treatment, eluents cannot restore the germination capacity of eluted pollen.

A diagram of the polyacrylamide gel electrophoretic pattern of proteins present in acetone protein precipitates of pollen eluents of Petunia hybrida redrawn from gel scans is presented in Figure 3.3. A gel scan is presented in Figure 3.4. A total of 19 individual bands was revealed by staining with Coomassie blue, indicating a strong diversity of protein components with respect to both charge and molecular weight. This conclusion is further supported by molecular weight range determinations of eluted proteins. Analytical procedures using liquid chromatography indicate a range in molecular weight between approximately 5,000 and 61,000 daltons. No sharp individual peaks were found, indicating a continual diversity in molecular weight between the above listed limits. No particular protein fraction constitutes a high percentage of the total.

Table 3.3

The Effects of Amino Acid-Protein and Carbohydrate Components of Dowex 50W-8X Effluents of Neutral and Acidic Fractions of Pollen Eluents of *Petunia hybrida* on Germination of Eluted Pollen Samples

Treatment	Germination (Per cent of Control)
Control	100
Eluted pollen	49.0
Neutral eluent fraction, 0.4 N NH_4OH Dowex 50W-8X effluent (carbohydrate component)	51.2
Neutral eluent fraction, 4.0 N NH_4OH Dowex 50W-8X effluent (amino acid-protein component)	80.9
Acidic eluent fraction, 0.4 N NH_4OH Dowex 50W-8X effluent (carbohydrate component)	53.2
Acidic eluent fraction, 0.4 N NH_4OH Dowex 50W-8X effluent (amino acid-protein component)	97.4

Values reported are per cents of control germinations. All germinations involved 25 mg pollen; in all cases eluent added is that quantity derived from 25 mg pollen. Values reported are means of 4 replications of three experiments. For each replication 400 pollen grains were counted.

Table 3.4

The Effect of Autoclaved Pollen Eluents of *Petunia hybrida* on Germination of Eluted Pollen Samples

Treatment	Germination (Per cent of Control
Control	100
Eluted pollen	47.6
Untreated eluent	74.1
Autoclaved eluent	42.6

Values reported are per cents of control germinations. All germinations involved 25 mg pollen; in all cases eluent added is that quantity derived from 25 mg pollen. Values reported are means of 4 replications of two experiments. For each replication 400 pollen grains were counted.

Figure 3.3. Discontinuous polyacrylamide gel electrophoresis of the acetone-precipitated protein fraction of 20 ml (2 min) eluents of pollen of Petunia hybrida. Developed at 2.5 milliamps per gel. Upper buffer tris-glycine (pH 8.5); lower buffer tris-HCl (pH 8.1). Redrawn from gel scan.

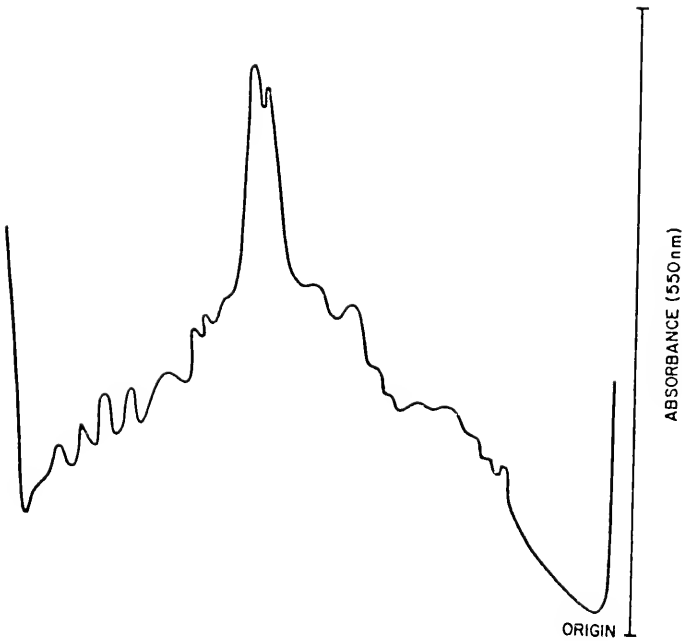

Figure 3.4. Gel scan of discontinuous polyacrylamide gel electrophoresis of acetone precipitated protein fraction of 20 ml (2 min) eluents of Petunia hybrida. Developed at 2.5 milliamps per gel and stained with Coomassie blue. Upper buffer tris-glycine (pH 8.5); lower buffer tris-HCl (pH 8.1).

Experiments 1, 2, and 3 testing the germination boosting capacities of molecular weight fractions of ammonium sulfate precipitated proteins greater than 10,000 daltons, greater than 50,000 daltons and greater than 50,000 but less than 100,000 daltons, respectively, are reported in Table 3.5. The results of these experiments indicate that proteins between 50,000 and 100,000 daltons appear to be responsible for increasing the per cent germination of eluted pollen.

When lyophilized 50,000 to 100,000 dalton fractions are taken up in protein extracting solution and applied to pre-charged polyacrylamide gels and subjected to electrophoresis under conditions identical to those used for electrophoresis of the total protein fraction, staining with Coomassie blue reveals only two bands (Figure 3.5). A gel scan is presented in Figure 3.6. This indicates that the 50,000 to 100,000 dalton fraction responsible for restoring the germination capacity of eluted pollen contains a maximum of two proteins. Since electrophoretic separation is based on charge as well as molecular weight, it cannot be concluded, however, that two molecular weight species are involved in restoring germination capacity. In addition, cellulase assays performed according to the procedure of Meyers et al. (1960), in which cellulase activity is measured in terms of glucose release, revealed no cellulolytic activity in the 50,000 to 100,000 dalton fraction.

Acetone precipitates of eluted pollen proteins contain approximately 16% carbohydrate, as determined by the phenol-sulfuric acid assay. The effect of varying quantities of acetone precipitated protein from total pollen eluents on the germination capacity of eluted pollen samples is presented in Figure 3.7. This experiment was undertaken to determine if germination restoring components are functioning in a

Table 3.5

Effect of Specific Molecular Weight Fractions of Ammonium Sulfate Precipitated Proteins of Eluents of Pollen of Petunia hybrida on the Germination of Eluted Pollen

EXPERIMENT 1: Effects of ammonium sulfate precipitated proteins greater than and less than 10,000 daltons on the germination of eluted pollen of Petunia hybrida

Treatment	Per cent Germination	Per cent of Control
Non-eluted pollen	51.8	100
Eluted pollen	33.5	64.7
Eluted pollen plus proteins greater than 10,000 daltons	52.4	101.3
Eluted pollen plus proteins less than 10,000 daltons	38.5	74.4

EXPERIMENT 2: Effects of ammonium sulfate precipitated proteins greater than and less than 50,000 daltons on the germination of eluted pollen of Petunia hybrida

Treatment	Per cent Germination	Per cent of Control
Non-eluted pollen	63.8	100
Eluted pollen	38.3	60.1
Eluted pollen plus proteins greater than 50,000 daltons	61.8	96.9
Eluted pollen plus proteins less than 50,000 daltons	40.6	63.7

Table 3.5
(Continued)

EXPERIMENT 3: Effects of ammonium sulfate precipitated proteins greater than 100,000 daltons and proteins less than 100,000 daltons but greater than 50,000 daltons on the germination of eluted pollen of *Petunia hybrida*

Treatment	Per cent Germination	Per cent of Control
Non-eluted pollen	64.8	100
Eluted pollen	40.6	62.7
Eluted pollen plus proteins greater than 100,000 daltons	41.6	64.2
Eluted pollen plus proteins between 50,000 and 100,000 daltons	54.8	84.6

Germinations performed for the above described experiments are reported as means of 4 replications of 2 experiments. For each replication 400 pollen grains were counted. Variations in values for the germination of eluted and control pollen is due to the diversity of pollen samples.

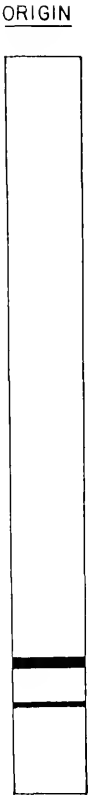

Figure 3.5. Discontinuous polyacrylamide gel electrophoresis of the protein fraction with molecular weight between 50,000 and 100,000 daltons, as prepared by ultrafiltration. Proteins were prepared from 20 ml (2 min) eluents of pollen of Petunia hybrida by precipitation with ammonium sulfate. Gels were developed at 2.5 milliamps per gel. Upper buffer tris-glycine (pH 8.5); lower buffer tris-HCl (ph 8.1). Redrawn from gel scan.

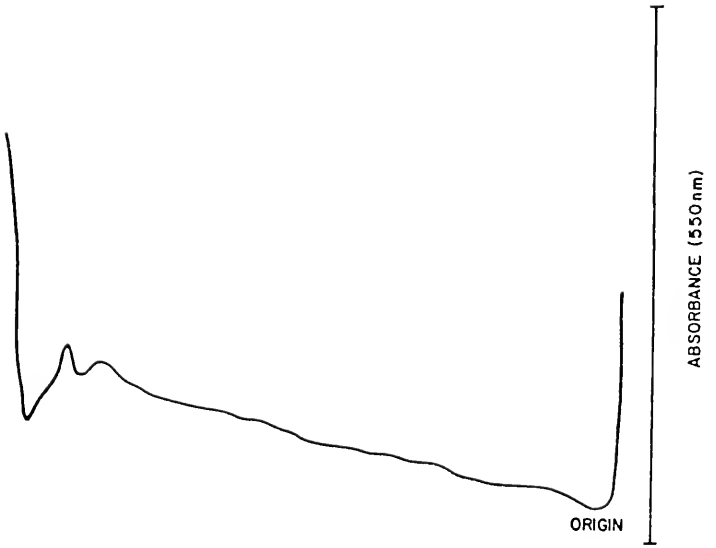

Figure 3.6. Gel scan of discontinuous polyacrylamide gel electrophoresis of the protein fraction with molecular weight between 50,000 and 100,000 daltons, as prepared by ultrafiltration. Proteins were prepared from 20 ml (2 min) eluents of pollen of Petunia hybrida by precipitation with ammonium sulfate. Developed at 2.5 milliamps per gel and stained with Coomassie blue. Upper buffer tris-glycine (pH 8.5); lower buffer tris-HCl (pH 8.1).

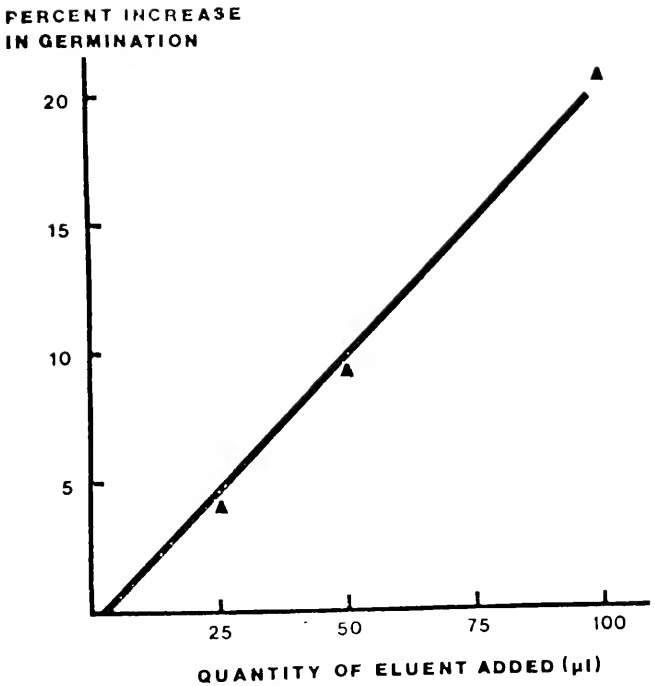

Figure 3.7. Effect of varying quantities of acetone-precipitated protein fraction of eluents of Petunia hybrida pollen on germination capacity of eluted pollen. Proetin precipitates from 125 mg pollen were redissolved in 500 µl germination medium and added to 25 mg eluted pollen samples prior to germination. 100 ml contains elutable proteins from 25 mg pollen. Values are averages of 4 experiments.

concentration independent manner, in which case their presence alone may result in the restoration of germination, or in a concentration dependent manner. One hundred twenty-five milligrams of pollen were eluted and proteins precipitated as described previously. The protein precipitate was redissolved in 500 µl pollen germination medium and varying amounts were added to 5 ml germination medium containing 25 mg eluted pollen. The resulting curve is a straight line. Linear regression analysis gives an equation of $y = 9.21x - 0.55$, with an r value of 0.998, indicating a good linear relationship between the per cent increase in germination of eluted pollen and the quantity of acetone precipitated protein added, within the range zero to the amount present in non-eluted pollen samples.

Discussion

Many factors have been implicated in controlling pollen germination, including calcium (Brewbaker and Kwack, 1963), boron (Schmucker, 1935), growth substances (Loo and Hwang, 1944; Bose, 1959; Raghavan and Baruah, 1959; Ermsweller et al., 1960; Vasil, 1960b; Carmichael, 1971), vitamins (Cooper, 1939; Vasil, 1960b), antibiotics (Vasil, 1960b), colchicine (Sen and Verma, 1963), inorganic ions, such as manganese, cobalt, sodium and potassium (Brink, 1924; Cooper, 1939; Yamada, 1958; Vasil, 1960b; Zielinski and Olez, 1963), and plant tissue extracts (Sen and Verma, 1963). The results presented here show that, in addition to the above listed factors controlling pollen germination, protein components contained within the pollen walls of Petunia hybrida are critical in controlling pollen germination in vitro.

After elution with water for 2 min the germination capacity of Petunia pollen is markedly decreased. However, the addition of eluted substances back to eluted pollen samples significantly restores germination capacity. The germination level of control pollen samples can be achieved by adding specific protein fractions back to eluted pollen samples. Since addition of acidic and neutral fractions of pollen eluents increases the germination acpacity, it is concluded that two or more components of the pollen eluents are likely responsible for restoration of germination capacity.

The addition of amino acid-protein and carbohydrate components of neutral and acidic fractions to eluted pollen shows that only protein-amino acid components possess the ability to increase germination of eluted pollen. These experiments lend evidence that neutral

and acidic proteins are involved. This contention is further supported by the fact that denatured eluent possesses no germination boosting capacity. In addition, there is a linear relationship between the added quantity of acetone precipitated protein prepared from pollen eluent (between the range zero to the amount present in non-eluted samples) and the capacity for germination of eluted pollen samples. The addition of larger quantities of eluent protein results in higher percentages of germination of eluted pollen. This indicates the possible direct involvement of eluted proteins in the germination process.

Gel electrophoresis of the protein fraction indicates a high diversity with regard to both size and charge. Nineteen individual bands were present (Figures 3.3 and 3.4). Electrophoresis of ammonium sulfate precipitates of pollen wall proteins of Cosmos bipinnatus (Howlett et al., 1975) revealed only six major fractions; preparative procedures used in Howlett's study eliminated proteins with molecular weights less than 10,000 daltons, while the present study included such low molecular weight components.

Molecular weight determinations support the electrophoretic observation of a diverse protein component of pollen walls of Petunia. Liquid chromatography of acetone protein precipitates yields a range of molecular weights. These ranges correspond to those obtained by Howlett et al. (1975) using SDS polyacrylamide gel electrophoresis to determine molecular weight. However, they found two major peaks at 11,500 and 30,000. Results with Petunia pollen proteins indicate a wide range in molecular weight with no major peaks, i.e., without a large percentage of the total protein compartmented within a narrow range of

molecular weight. This is clearly seen on polyacrylamide gels (Figures 3.3 and 3.4), where electrophoretic separation on the basis of both charge and molecule size reveals many components.

Howlett et al. (1975) reported two faint PAS-staining bands after electrophoretic separation of pollen wall proteins of Cosmos bipinnatus. Techniques for localizing carbohydrate-containing proteins on polyacrylamide gels of Petunia pollen proteins have been unsuccessful, which may indicate the absence of glycoproteins. However, analysis of the total protein fraction of Petunia eluents indicated that it contains approximately 16% carbohydrate. This value agrees favorably with the value of 14.4% carbohydrate reported for short ragweed allergen (Underdown and Goodfriend, 1969). If glycosylation is a prerequisite for secretion, as Eylar (1965) has suggested, it is logical that protein components synthesized in the microsopre protoplast and deposited extracellularly in wall sites are glycoproteins.

Preparation of 10,000, 50,000 and 100,000 dalton fractions by ultrafiltration of ammonium sulfate precipitated proteins of Petunia pollen eluents and addition of these fractions to samples of eluted pollen germinating in vitro have shown that proteins between 50,000 and 100,000 daltons are likely responsible for resotring the germination of eluted pollen to control levels. Electrophoresis of this fraction yields only two bands indicating that the germination restoring capacity is contained in one, or possibly two, molecular species. Table 3.5 reports that eluted protein fractions derived from 25 mg pollen and within the molecular weight range of 50,000 to 100,000 daltons boost the germination of 25 mg samples of eluted pollen to 83.9% of the controls. Separation techniques, especially a two-step procedure as was

used to prepare the 50,000-100,000 dalton fraction, result in some loss of protein. Also, it should be kept in mind that the germination restoration is dependent on the amount of the protein fraction added (Figure 3.4). Protein loss during preparative procedures is likely to contribute to the seemingly low value for per cent germination of eluted pollen plus the 50,000 to 100,000 dalton fraction.

Since the addition of a fairly narrow range molecular weight fraction can significantly restore the germination capacity, which was decreased by elution, such factors may also be controlling germination of Petunia pollen in vivo. Both acidic and neutral proteins appear responsible. Since proteins active in germination restoration are heat-labile, an enzymatic nature may be indicated. Possible candidates for these proteins would include enzymatic proteins whose molecular weights are within the range found to be active in boosting the germination of eluted pollen. The possibility of the cellulase complex (molecular weight range 52,000 to 76,000 daltons) being a key factor in the germination of Petunia pollen is attractive, since softening of the pectocellulosic intine layer in pore regions of pollen grains is required for pollen tubes to emerge. Stanley and Search (1971) found considerable cellulase activity in pollen eluents of pine, cattail and pear. However, assays for cellulase activity, measured as the release of glucose from carboxymethyl cellulose, have shown no cellulase activity in the 50,000 to 100,000 dalton fraction which promotes pollen germination.

The work of Heslop-Harrison, Knox and co-workers has revealed a possible function for diffusible proteins of pollen grains as "recognition substances" in both interspecific (Knox et al., 1972) and

intraspecific incompatibility (Howlett et al., 1975), although Fett et al. (1976) have not been able to find such "recognition substances" contributing to gametophytically controlled self-incompatibility in Lilium longiflorum. The only available study somewhat comparable to the work presented here focussed on components of pollen eluents of Cosmos bipinnatus (Howlett et al., 1975). Cosmos, a member of the Compositae, has trinucleate pollen and a sporophytic incompatibility system. The incompatibility reaction takes place on the surface of the stigma within 30 min of pollination. On the other hand, Petunia, a member of the Solonaceae, has binucleate pollen and a gametophytically controlled incompatibility system. The incompatibility reaction takes place after the pollen grain has germinated and the pollen tube has entered the style. Pollen of Petunia hybrida germinates easily on compatible or incompatible stigmas, regardless of the presence of elutable components of the pollen wall (Chapter Two). These results are similar to those obtained with Lilium (Fett et al., 1976). However, elutable components of Petunia pollen appear intimately involved with germination in vitro.

Both carbohydrate and protein components of eluents of Petunia pollen are diverse. It is interesting that carbohydrate and protein components of pollen eluents of Cosmos are considerably less diverse. Since in Cosmos the acceptance or rejection of a given pollen grain must take place quickly and "recognition substances" are likely involved, it is possible that a lack of diversity of pollen eluent components in Cosmos indicates directed synthesis of specific molecules required for the recognition reaction.

In Cosmos elutable proteins which are able to partially overcome self-incompatibility are heat stable (Howlett et al., 1975). In pollen of Petunia heat-labile proteins appear intimately involved in germination in vitro. Their absence results in poor germination ability, which can be restored by the addition of eluted proteins. This adds further support to the hypothesis that protein components of the walls of pollen grains of Petunia and Cosmos are functionally distinct.

CONCLUSIONS

Analysis of the data concerning the quantities of carbohydrates, proteins and total elutable components of pollen wall eluents of 42 angiosperm species forces the conclusion that greater amounts of all of the above constituents are found in eluents of binucleate pollen species. A closer look at pollen eluents of Petunia, a species with binucleate pollen and whose physiology and genetics are fairly well known, revealed a diverse carbohydrate component, including the presence of several free monosaccharides and additional monosaccharide components of oligosaccharides. It is likely that such carbohydrates diffuse from "storage areas" of the pectocellulosic intine layer of the pollen wall, or are products of the breakdown of tapetal cells and are stored within the exine. Since some degree of similarity has been noted between these carbohydrates and components of the transmitting tissue, a possible nutritive function for these carbohydrates during early pollen tube growth must be considered.

The observation that after elution pollen of Petunia germinates poorly in vitro and that germination of eluted pollen can be restored by the addition of eluted substances posed the question of which components of Petunia pollen eluents were responsible for restoring germination. Addition of the carbohydrate fraction of eluents to eluted pollen germinating in vitro was not able to restore germination. However, this does not indicate that carbohydrates of pollen eluents

are not serving roles as metabolic substrates during early pollen tube growth on the stigma, but merely indicates the involvement of another factor(s) in restoring pollen germination *in vitro*. This factor turned out to be a heat-labile protein fraction, without detectable cellulase activity, and with a molecular weight between 50,000 and 100,000 daltons. Since this fraction is a key in the control of germination *in vitro*, it is also likely to be intimately involved in germination on the stigma. Therefore, further work is suggested to identify the precise nature of this fraction in order to gain a more thorough knowledge of the process of pollen germination and fertilization.

When comparing the analytical work described here, utilizing eluents from a species having binucleate pollen, with a somewhat similar study of pollen diffusates of a species having trinucleate pollen (*Cosmos*), the most striking differences are seen in the diversity of eluent or diffusate components. *Petunia* eluents contain higher diversity with regard to both carbohydrate and protein components. Clearly, no phylogenetic trends can be based on studies of only two species, especially when analytical techniques used in determining characteristics of eluents in the two studies differed somewhat. However, it may be boldly suggested that elutable compounds from phylogenetically primitive binucleate pollen species are not only greater in quantity than those of species with trinucleate pollen, but may also contain more diversity. Eluents of trinucleate pollen may have been derived by reduction from the primitive binucleate condition and may reflect synthesis of only specific molecules mediating sporophytically controlled incompatibility responses.

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BIOGRAPHICAL SKETCH

Edward George Kirby, III was born 28 November, 1947, in Toledo, Ohio. He attended elementary school in Birmingham, Michigan, and graduated from Detroit Country Day School in 1965. He pursued a Bachelor of Science degree in zoology at the University of Michigan and was graduated in 1969. In addition, he received a secondary teaching certificate in biology at that time.

He first attended the University of Florida in the fall of 1969 and was awarded the Master of Science degree in botany in 1971.

During the interim between his master's and doctoral studies he spent two periods at the Botanisch Laboratorium, Universiteit Nijmegen in Holland where part of his dissertation research was performed. Currently he is a post-doctoral research associate at the Tree Genetics Laboratory, Oregon Graduate Center, Beaverton, Oregon.

Edward G. Kirby, III is married to the former Melinda Sears Moody.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Indra K. Vasil, Chairman
Professor of Botany

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Willard W. Payne, Co-chairman
Professor of Botany

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Henry C. Aldrich
Professor of Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ray E. Goddard
Professor of Forest Resources and Conservation

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Rex L. Smith
Associate Professor of Agronomy

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June 1977

Jack L. Fry
Dean, College of Agriculture

Dean, Graduate School